Application No.: 10/561119
Response to Non-Final Office Action dated 06/23/08

Customer No.: 000027683
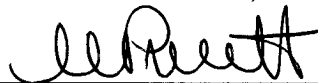Atty. Docket No.: 36697.17

EXHIBIT A

Certificate of Service

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Margaret A. Pruitt

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Prickett et al	
App. No	:	10/561,119	
Filed	:	June 17, 2004	
For	:	ASSESSMENT OF SKELETAL GROWTH USING MEASUREMENTS OF NT-CNP PEPTIDES	
Examiner	:	James L Grun	
Art Unit	:	1641	

CERTIFICATE OF MAILING

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450, on

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(Date)

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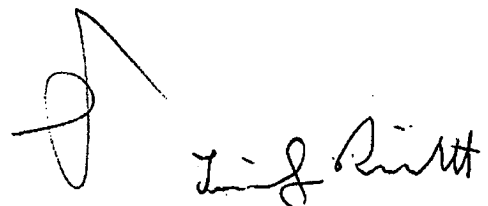Mark Moore

DECLARATION OF TIMOTHY PRICKETT
UNDER 37 C.F.R. §1.132

Commissioner of Patents
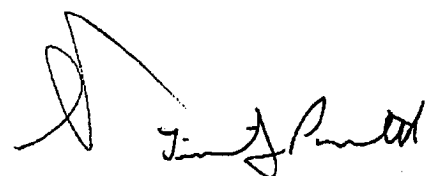P O Box 1450
Alexandria, VA 22313-1450

Dear Sir

I, Timothy Charles Ramsey Prickett, declare as follows:

1. I am a current employee at Otago University, having its principal place of business at Otago House, 481 Moray Place, Dunedin, New Zealand.

2. I am a co-inventor of the above-captioned application ("the present application").

3. I understand that an objection has been raised under 35 USC §112 in the Office Action mailed on June 23, 2008 that the term "biological sample" is not supported by the description. I further understand that this term has been amended to recite "biological fluid".
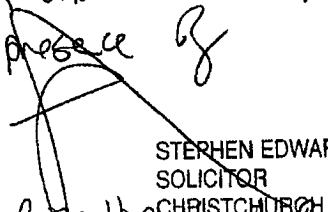
4. I have carried out experiments that show that NT-CNP can be detected in biological fluids, other than plasma, and in particular, in urine. Figure 1, which shows NT-CNP levels in neonatal urine (top panel) and in pooled adult urine (bottom panel) is attached as exhibit TCRP 1. Details of how this experiment was carried out are set out in Appendix A, attached as exhibit TCRP6. This experiment shows that the level of NT-CNP can be correlated with skeletal status as much higher levels are found in samples taken from children than from adults.

5. This Declaration is submitted to show that NT-CNP is detectible in biological fluids other than plasma, and that the level of NT-CNP in biological fluids, correlates to skeletal status.

6. I conducted an experiment whereby NT-CNP and alkaline phosphatase plasma levels were measured in adult sheep after administration of estrogen. Estrogen is known to stimulate bone formation. The results are shown in figure 2, attached as exhibit TCRP2, and show that both alkaline phosphatase and NT-CNP are elevated in adult sheep in response to estrogen.

7. This Declaration is submitted to show that the level of NT-CNP can be used to measure the impact of agents capable of affecting the skeletal status of adults.

8. I conducted a further experiment using a well established model of osteo-arthritis in adult dogs. The results are shown in figure 3, attached as exhibit TCRP3. A significant increase in NT-CNP occurs three months after induction of focal articular cartilage trauma. The increase in NT-CNP reflects chrondocyte proliferation in response to injury.

9. This Declaration is submitted to show that the level of NT-CNP can be used to measure the impact of bone disease or disorder capable of affecting the skeletal status of adults.

10. I understand that a number of prior art documents have been cited under 35 USC§103 against the claims of the present application. I have read these documents and the examiner's comments in the Office Action.

11. The examiner contends that it would have been obvious from the prior art that circulating NT-CNP levels could be correlated with skeletal growth. I must disagree. It was surprising at the date of the invention that circulating NT-CNP levels could be correlated with skeletal growth in neonatal, childhood, pubertal as well as adult subjects (as shown in the ROC curve in figure 4, attached as exhibit TCRP 4).

12.   I also tested plasma NT-CNP levels in a sub group of children with extreme short stature and found very **high** levels of NT-CNP in these children who, I am informed, have very **low** skeletal growth rates. This subgroup have a block to the action of CNP within the bone growth plate and are now able to be diagnosed by the high NT-CNP level. These results are shown in figure 5, attached as exhibit TCRP5. This figure also shows high NT-CNP levels in subjects with distorted bone overgrowth associated with chromosomal abnormalities. Again, NT-CNP levels have, for the first time, been used to diagnose this disorder together with X-rays showing patterns of bone overgrowth.

13.   This Declaration is submitted to show that it was surprising over the prior art that NT-CNP levels can be used to determine skeletal status in children as well as adults, and to diagnose diseases and disorders that affect skeletal growth. The prior art merely taught that NT-CNP levels were associated with cardiovascular disease (*Prickett et al*); degradation products of CNP were associated with cardiovascular disease (*Buechler et al*); CNP stimulates bone growth (*Yasoda et al*) and CNP acts locally to induce bone growth (*Chusho et al*). None of the cited art, either alone or in combination teaches the use of NT-CNP to assess skeletal growth.

14.   I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or patent issuing therefrom.

Date: 12\11\08

By: _____

Timothy Charles Ramsey Prickett

Solemnly & Sincerely declared by
Timothy Charles Ramsay Prickett
in the presence of

STEPHEN EDWARD BRAY
SOLICITOR
CHRISTCHURCH New Zealand

Solicitor of the High Court

1690039_1_Declaration.DOC

This is the exhibit marked "**TCRP1**"
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this 12 day of Nove K 2008 before me

. . . .
A Solicitor of the High Court of New Zealand

STEPHEN EDWARD BRAY
SOLICITOR
CHRISTCHURCH

Application No.: 10/561119
Response to Non-Final Office Action dated 06/23/08

Customer No.: 000027683
Atty. Docket No.: 36697.17

Should the Examiner have any questions, a telephone call to the undersigned Applicants'

representative would be appreciated.

Respectfully submitted,

Mark D. Moore, Ph.D.
Registration No. 42,903

Dated: November 24, 2008
HAYNES AND BOONE, L.L.P.
Telephone: 713-547-2040
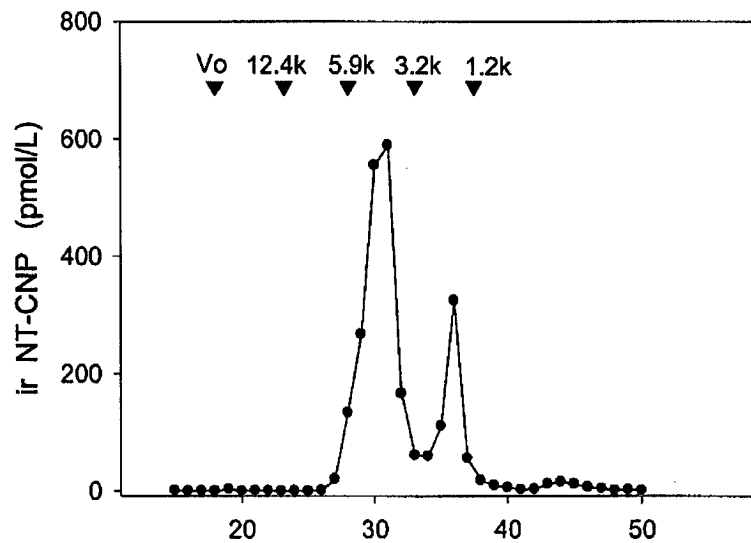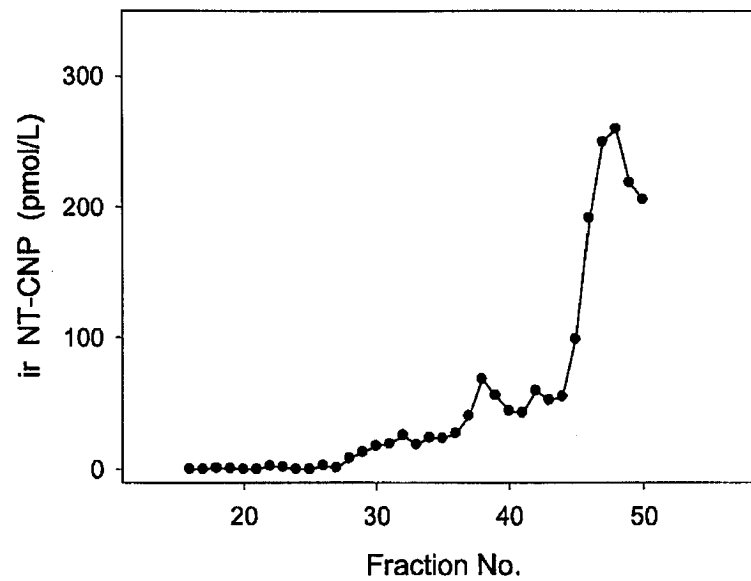Facsimile: 214-200-0853

36697.17
H755809

A

B

Figure 1

Figure 1 is a graph showing the results from size exclusion HPLC of extracts of A) urine from neonate less than one month old, and B) pooled adult urine. NT-CNP should elute between fractions 29-31. Column void and elution positions of molecular markers are shown by arrows.

This is the exhibit marked "**TCRP2**"
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this   1L   day of   March   2008 before me

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A Solicitor of the High Court of New Zealand

STEPHEN EDWARD BRAY
SOLICITOR
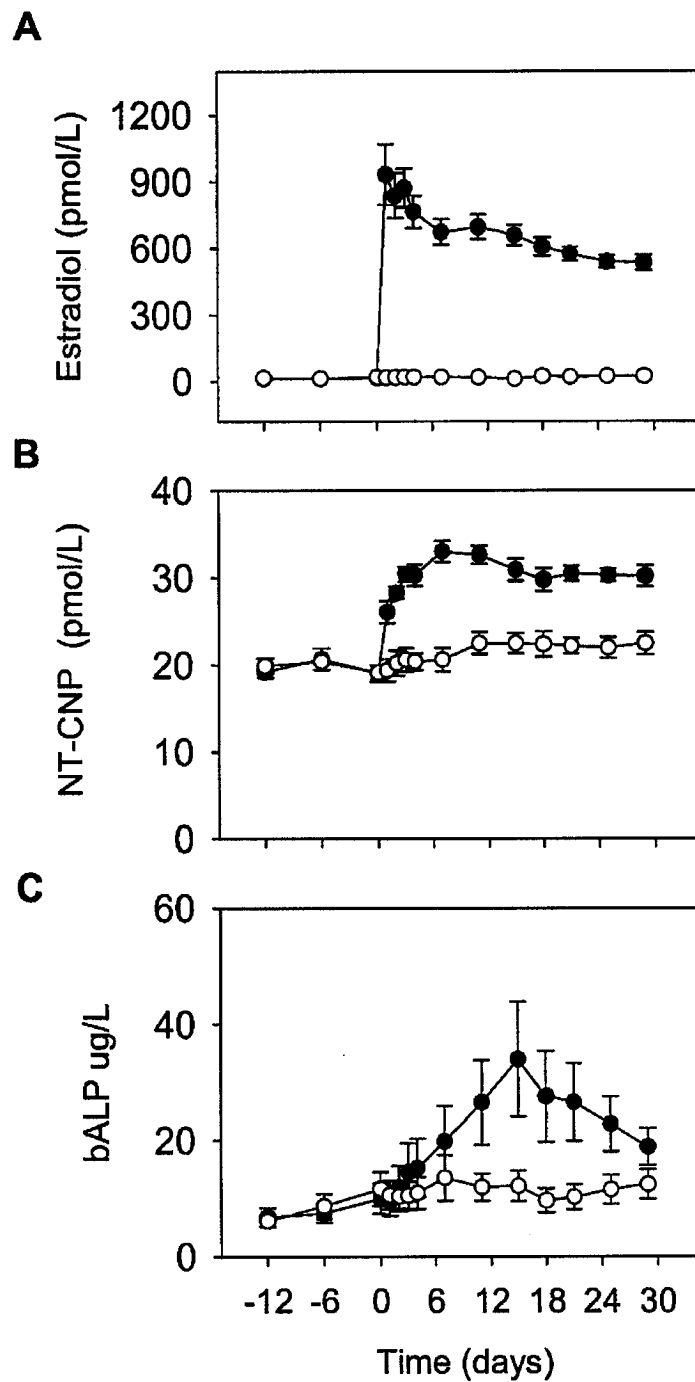CHRISTCHURCH

A

B

C

Figure 2

Figure 2 shows the effect of estradiol implants (17ug/kg/day release rate, filled circles, n=8) or control vehicle (open circles, n=8) administered to adult ewes. Responses in plasma estradiol (A), NT-CNP (B) and bone specific alkaline phosphatase (C) are shown. Values are mean +/- SEM.

This is the exhibit marked "TCRP3"
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this 12 day of November 2008 before me

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A Solicitor of the High Court of New Zealand
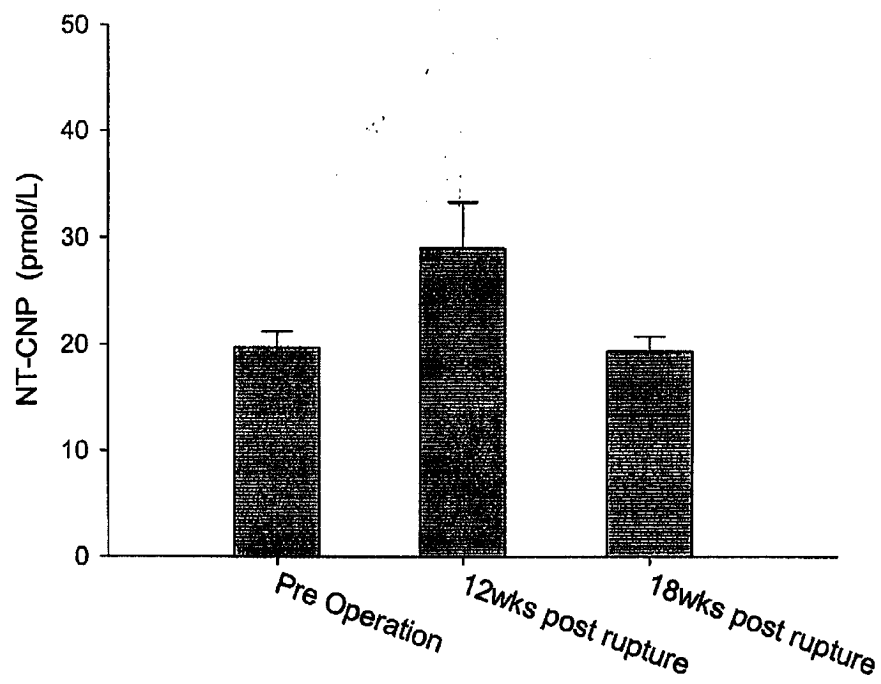
STEPHEN EDWARD BRAY
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CHRISTCHURCH

Figure 3

Figure 3 shows the plasma NT-CNP response to anterior cruciate ligament rupture (right foreknee) induced by monopolar radio frequency energy at arthroscopy. Results are mean values (n=6 adult dogs) +/- SEM.

This is the exhibit marked **"TCRP4"**
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this ⟨12⟩ day of ⟨November⟩ 2008 before me

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A Solicitor of the High Court of New Zealand
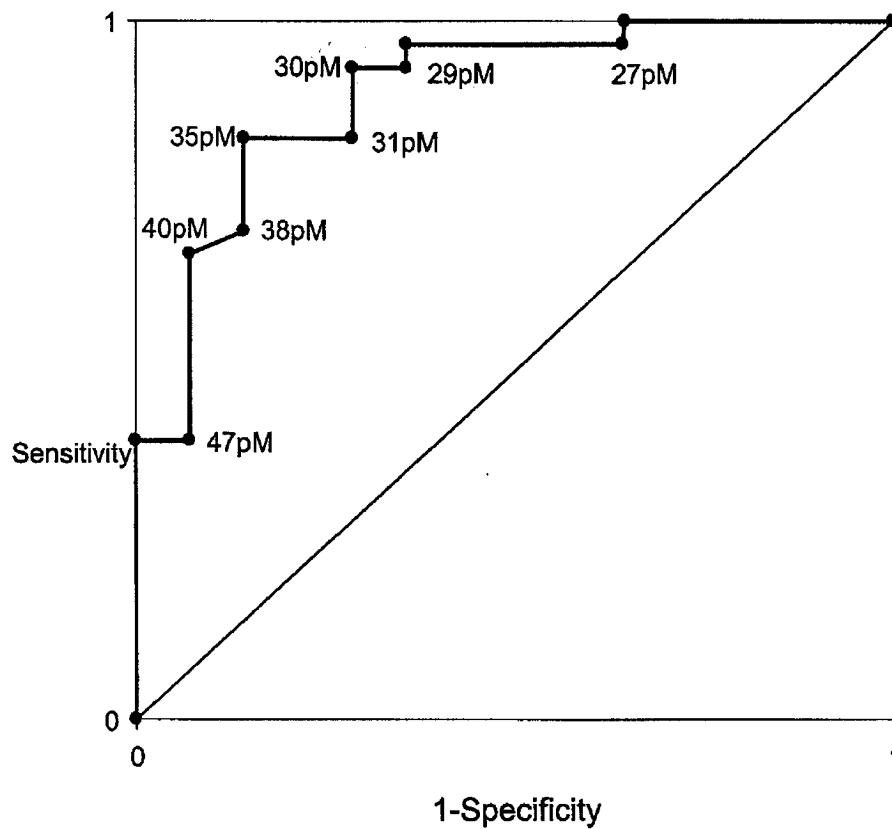
STEPHEN EDWARD BRAY
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Figure 4

Figure 4 shows a receiver operator curve (ROC) for the different cut off values of plasma NT-CNP concentration used to predict cessation of linear growth in children and adolescents. Cessation of growth is defined as a growth velocity of less than 2 cm per year.

This is the exhibit marked "**TCRP5**"
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this 12th day of November 2008 before me

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A Solicitor of the High Court of New Zealand
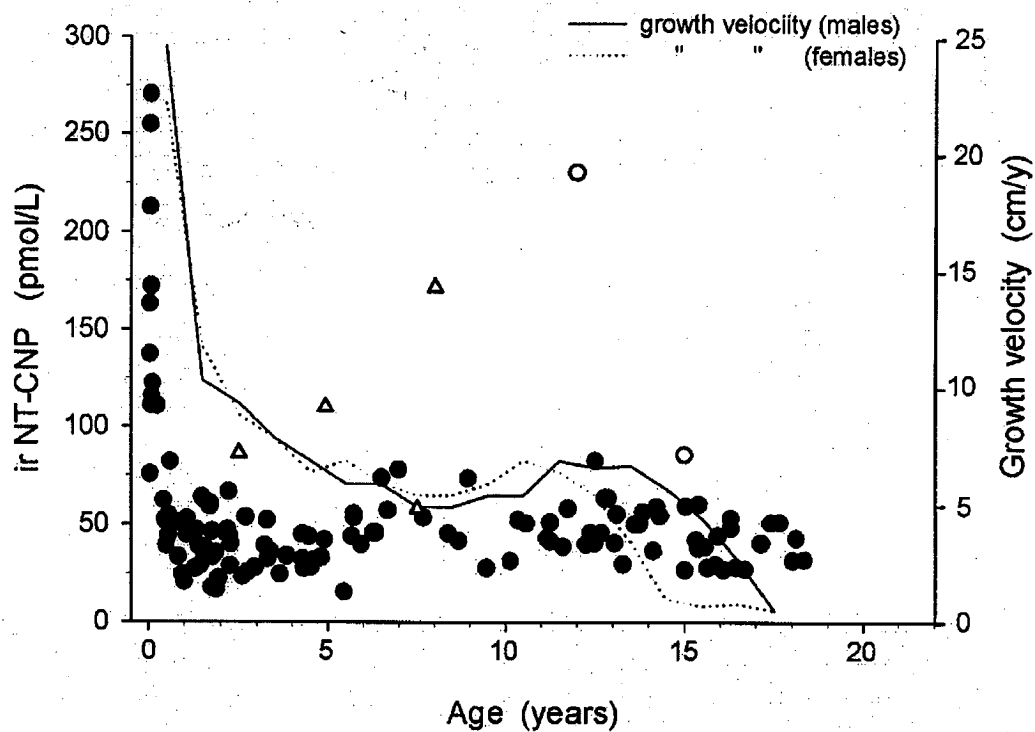
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Figure 5

Figure 5 is a graph showing immunoreactive plasma NT-CNP concentrations in blood obtained from individual children aged 0 – 18 years old. Filled symbols are from children attending hospital clinics, open circles represent children who over-express CNP due to chromosomal rearrangements and open triangles patients with Acromesomelic dysplasia Maroteaux type. The solid and dotted lines show normal median age- related growth velocities from the published literature for male and female children respectively.

This is the exhibit marked "**TCRP6**"
annexed to the Declaration of **Timothy Charles Ramsey Prickett** affirmed at Auckland
this   12th   day of   November   2008 before me

....

A Solicitor of the High Court of New Zealand

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SOLICITOR
CHRISTCHURCH

APPENDIX A

Materials and Methods Used in Experiments (Fig 1-5)

Figure 1. NT-CNP is present in neonatal urine but not in urine from adults.
Urine (25ml volume) obtained from an infant (< than one month of age) whose linear growth was rapid, and from a sample (25ml volume) of pooled urine obtained from normal healthy adults whose linear growth had ceased, were analysed.

Urine samples (4-5ml) were extracted using Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts, USA) prewashed with 5ml methanol and 5ml 0.1% trifluroacetic acid. Urine was run slowly through the column, which was then washed with 5ml 0.1% trifluroacetic acid. NT-CNP was then eluted with 80% isopropanol in 0.1% trifluroacetic acid and dried under an air stream at 37°C after addition of 10ul of 1% triton X100. Extracts were re-suspended in 20% CH3CN in 0.1% TFA prior to size exclusion HPLC.

RIA for N-terminal CNP: NT-CNP(1-15)-Tyr^{16} (5μg) was iodinated using 0.5mCi Na^{125}I in the presence of 10μg chloramine-T in 5μl 0.5M phosphate buffer, pH 7.5 for 30 seconds, followed by the addition of 50μg cysteine, 25μg BSA and 20μg KI in 100μl buffer. The resulting mixture was loaded onto a 10cm RP300 Brownlee column and eluted with a gradient from 0-60% acetonitrile in 49mM KH$_2$PO$_4$ pH 2.9 over 30 min at 1ml/min, collecting 0.5ml fractions. The fraction containing the major peak of radioactive NT-CNP(1-15)-[^{125}I]Tyr^{16} was used in the RIA. Peptide standards were made from synthetic human NT-CNP(1-19) taking into account the purity data supplied. All standards, sample extracts, antisera and tracer solutions were made up in assay buffer consisting of 0.1% bovine serum albumin, 0.01% sodium azide, 0.1% triton X100, and 0.05% sodium chloride in 0.1M phosphate buffer pH 7.4. 200μl of sample extract or 7-38,000 pM NT-CNP(1-19) standard (all in duplicate) were pre-incubated with 100μl antiserum solution at a dilution of 1:6000 for 22h at 4°C prior to the addition of 100μl tracer solution (NT-CNP(1-15)-[^{125}I]Tyr^{16}) containing 2000 cpm for a further 24h at 4°C. Bound and free NT-CNP(1-15)-[^{125}I]Tyr^{16} were separated by a solid phase second antibody method (Sac-cell, Donkey-Anti Rabbit, IDS Ltd, England).

RIA combined with size exclusion HPLC analysis showed the major immunoreactive NT-CNP peak in the neonatal urine samples had a molecular weight close to 5 kDa (fractions 30-31, Figures 1 A). These results show the presence of NT-CNP fragment(s) in neonatal urine. No detectable peak at fraction 30 (Figure 1 B) was identified in the pooled adult urine sample. Thus the difference in growth and skeletal status between neonates and adults is reflected by the excretion of NT-CNP in the urine.

Figure 2. NT-CNP can be used to measure the impact of agents capable of affecting skeletal status in adults.
Estrogen is known to stimulate bone formation in adults. I studied the effect of estrogen on NT-CNP and bone specific alkaline phosphatise (bALP, a marker of new bone growth) in adult sheep.

Sixteen anoestrous adult ewes (aged > 3 years) were randomly allocated to receive either estradiol (17μg/kg per day, Compudose 400 implants, n=8,) or sham implants (n=8). Blood samples were drawn prior to the intervention and then at intervals of 1-4 days for 30 days for measurement of plasma estradiol, NT-CNP and bone specific alkaline phosphatase. The results (Figure 2) show that both bALP and plasma concentrations of NT-CNP are elevated in adult sheep in response to estrogen. Plasma NT-CNP can be used as a marker of the response of the adult's skeletal status to agents affecting bone formation.

Figure 3. NT-CNP reflects arthritis -induced changes in cartilage in adults.
It is well recognised that osteo-arthritis causes focal damage to the affected articular cartilage which is associated with a later phase of focal chondrocyte proliferation. Using samples from a study employing a well established animal model of mild osteo-arthritis, I measured the changes in plasma NT-CNP collected before and after joint trauma in 6 adult dogs. In each dog, mild histological changes of osteo-arthritis developed in the right fore knee following rupture of the anterior cruciate ligament induced by mono-polar radio frequency energy at arthroscopy. A significant increase in plasma NT-CNP was found at 12 weeks post rupture, compared to pre-surgery levels. These results show that a change in an adult's skeletal status (in this case the development of osteo-arthritis) is associated with a change in plasma NT-CNP.

Figure 4. Predicting cessation of linear growth.
Plasma NT-CNP predicts not only the skeletal growth of a pre-adult but also the attainment of final adult height of an individual subject. Plasma obtained from 44 subjects (aged 2 – 18 years) was extracted on Sep Pak columns and assayed for NT-CNP. The growth velocity of these subjects was also measured in the subsequent 3-6 months. The predictive value of a plasma NT-CNP concentration in assessing growth potential was examined by receiver operator curve analysis (Figure 4). Growth cessation (growth velocity less than 2cm per year) was correctly predicted in 80% of the subjects exhibiting a plasma NT-CNP less than 35 pmol/L (sensitivity 80%, specificity 86%). The area under the curve (AUC) was 0.90. In healthy pre-adults, plasma NT-CNP can be used to determine the attainment of final adult height.

Figure 5. Use of NT-CNP in the diagnosis of abnormal skeletal growth.
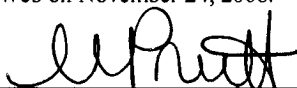Plasma samples were obtained from 105 individual children (aged 0-18years) attending hospital clinics, the concentration of NT-CNP measured and then plotted according to chronological age at the time of sampling. Plasma was also obtained from each of 4 children with the diagnosis of Acromesomelic dysplasia Maroteaux type. These children have extreme short stature and low growth rate due to a mutation in the gene coding for the CNP receptor. The concentrations of NT-CNP in these children are elevated when the plotted values are viewed in relation to the age-matched reference population. Plasma was also collected from two additional subjects with distorted bone overgrowth associated with chromosomal rearrangements. Plasma NT-CNP concentrations, when plotted according to chronological age at the time of sampling, were abnormally elevated when compared to the reference population.
These surprising results are the first to show that NT-CNP concentrations in plasma have the power to distinguish and diagnose specific disorders of skeletal growth when assessed according to an age-based reference range, and concurrent growth velocity.

Application No.: 10/561119
Response to Non-Final Office Action dated 06/23/08

Customer No.: 000027683
Atty. Docket No.: 36697.17

EXHIBIT B

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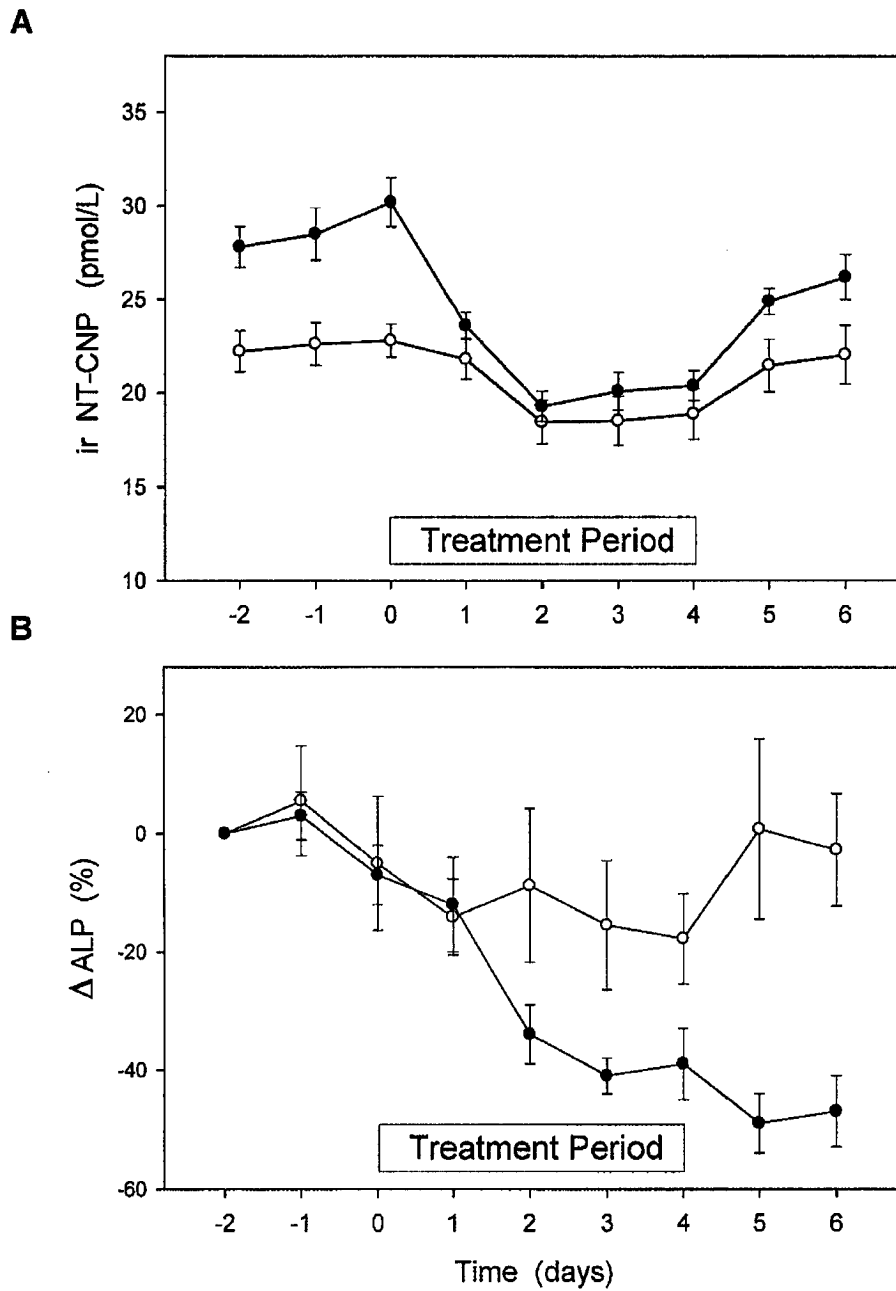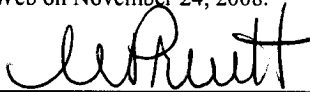Margaret A. Pruitt

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A

B

Figure 6A + 6B.

Effect of 4 days of treatment with a glucocorticoid (dexamethasone, 0.25mg/kg/day) in 15 week old lambs (filled circles, n=8) and in adult sheep (open circles, n=8) on (A) plasma NT-CNP and (B) changes (Δ) in plasma alkaline phosphatase activity (ALP). Values are mean ± SEM;

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Customer No.: 000027683
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EXHIBIT C

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Margaret A. Pruitt

Amino-Terminal proCNP: A Putative Marker of Cartilage Activity in Postnatal Growth

TIMOTHY C.R. PRICKETT, ADRIENNE M. LYNN, GRAHAM K. BARRELL, BRIAN A. DARLOW,
VICKY A. CAMERON, ERIC A. ESPINER, A. MARK RICHARDS, AND TIMOTHY G. YANDLE

*Department of Medicine [T.C.R.P., V.A.C., E.A.E., A.M.R., T.G.Y.], Christchurch School of Medicine and
Health Sciences, Christchurch 8015, New Zealand; Department of Pediatrics [A.M.L., B.A.D.],
Christchurch Hospital, Christchurch 8020, New Zealand; and Agricultural and Life Sciences Division
[G.K.B.], Lincoln University, Christchurch 8150, New Zealand*

ABSTRACT

Recent evidence from rodents and humans shows that C-type natriuretic peptide (CNP) plays an essential role in endochondral bone growth. We recently identified a stable product of proCNP, amino-terminal proCNP (NT-proCNP), which unlike CNP is readily measurable in human and ovine plasma. Hypothesizing that plasma NT-proCNP concentrations reflect in part CNP synthesis within growth plates of rapidly growing cartilage, we studied levels of CNP forms in both children and lambs and related these to age, growth velocity, and biochemical markers of bone turnover. Plasma NT-proCNP levels were elevated at birth and fell progressively with age. Significant associations between plasma NT-proCNP and height velocity, alkaline phosphatase, and type 1 collagen C telopeptide were identified in children (aged 5–18 y). In longitudinal animal studies, elevated plasma concentration of NT-proCNP in 1-wk-old lambs fell progressively to mature adult levels at age 27 wk. Plasma NT-proCNP showed a highly significant association with alkaline phosphatase and metacarpal growth velocity. Glucocorticoids, a treatment known to inhibit cartilage proliferation, reduced metacarpal growth elongation in 4-wk-old lambs and markedly lowered circulating NT-proCNP levels during the treatment period. In summary, NT-proCNP levels in blood show a strong association with growth velocity and markers of bone formation and may well serve as a useful marker of growth plate activity in humans and other mammals. (*Pediatr Res* 58: 334–340, 2005)

Abbreviations
ALL, acute lymphatic leukemia
ALP, alkaline phosphatase
CNP, C-type natriuretic peptide
CV, coefficients of variation
NT-proCNP, amino-terminal pro C-type natriuretic peptide

Skeletal growth results from a coordinated sequence of events involving proliferation and hypertrophy of chondrocytes within growth plates of long bones and deposition of intercellular matrix (1). Regulation of this process (endochondral growth) is complex and dependent on numerous paracrine and endocrine factors that variably modulate growth *in utero* and during the postnatal growing period. Despite increasing knowledge of the importance of paracrine factors, including bone morphogenetic proteins, Indian hedgehog, fibroblast growth factor, and PTH-related peptide, none of these has yet been used to assess growth plate activity in the clinical setting.

Atrial (ANP), B-type (BNP), and C-type natriuretic peptides (CNP), each the product of a separate gene, constitute a family

of structurally related peptides that regulate blood pressure and volume homeostasis (2). CNP, the most conserved across species of the three hormones, in contrast to ANP and BNP is not readily detected in blood, and circulating CNP has minimal natriuretic or vasodepressor activity in humans (3). Acting *via* its specific guanylyl cyclase receptor NPR-B (4), CNP has antiproliferative actions in vascular tissue (5), which may serve to regulate vascular remodeling and regeneration (6). Whereas the role of CNP in cardiovascular health is still unclear, several recent lines of evidence indicate that CNP plays an essential part in endochondral growth. CNP transcripts and their receptor NPR-B are expressed in chondrocytes of growth plates (7), and the addition of CNP stimulates both proliferative and hypertrophic zones, elongating bone explants in tissue culture (8). Furthermore, disruption of the CNP gene in mice yields a dwarfed phenotype and reduced chondrocyte proliferation, which can be rescued by targeted re-expression of the gene (9). That CNP is also essential to endochondral growth in humans was documented recently by the finding that loss-of-function mutations in NPR-B cause the profoundly dwarfed phenotype

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of acromesomelic dysplasia, Maroteaux type (10). Taken together, these findings confirm that CNP gene expression and action are important regulators of skeletal growth in mammals, including humans.

In light of similar patterns of processing of proANP and proBNP yielding bioactive carboxy-terminal and the (inactive) amino-terminal forms [NT-proANP (11) and NT-proBNP (12), respectively], we sought and successfully identified (13) a stable product of the CNP gene, amino-terminal proCNP (NT-proCNP), which unlike CNP is readily measurable in human plasma. Hypothesizing that plasma NT-proCNP concentration reflects in part CNP synthesis within growth plates of rapidly growing cartilage, we studied levels of CNP forms in both children and growing lambs and related these to age and growth velocity. Here we report for the first time that NT-proCNP levels in plasma are inversely related to age in both humans and sheep and correlate with growth velocity and markers of bone turnover before and after glucocorticoid administration, a treatment that is known to inhibit chondrocyte proliferation and linear growth.

METHODS

Adults. Blood samples for measurement of CNP and NT-proCNP were drawn from 16 healthy adult volunteers (aged 20–60 y) and 101 normal healthy adults (aged 50–80 y) who were recruited by random selection from the local electoral roll.

Children. Venous umbilical cord plasma was obtained from five full-term and eight premature (<32 wk gestation) deliveries. To define age- and growth-related changes in plasma CNP forms, we recruited an additional 60 children (33 female, 27 male), aged 5–18 y, from hospital outpatient clinics. All had normal renal function, and none showed cardiac abnormalities. Reasons for clinic attendance included monitoring of growth and development (32 patients), obesity (five patients), adrenal disorder (four patients), chronic inflammatory states (five patients), and history of oncologic disease (nine patients). Two of the last group had recently commenced chemotherapy and high-dose glucocorticoids for acute lymphoblastic leukemia (ALL) 1 wk before study. Four of the remaining 58 children had also received glucocorticoids (three physiologic replacement doses for adrenal insufficiency and one on a reducing dose of prednisone 10–20 mg daily for dermatomyositis). Of the total group (n = 60), 21 were receiving no medication at the time of study. In all recruited children, height (Harpenden Stadiometer) and weight were measured and blood was drawn on the same day (between 1300 and 1600) for CNP forms, alkaline phosphatase (ALP), and type 1 collagen C telopeptide. Using heights measured at an interval of at least 3 mo and within 6 mo of blood sampling, it was possible to determine concurrent growth velocity in 23 of the 60 children. All studies were approved by the Canterbury Ethics Committee, and informed written consent was given by all participants or their parents, as appropriate.

Effect of age in sheep. Twelve sets of healthy mixed-sex Coopworth twin lambs were studied for a period of 6 mo. Lambs and mothers were maintained on pasture until weaning at 12 wk. From 1 wk of age, lambs (n = 24) were weighed, the left metacarpal length was measured (vernier calliper), and jugular blood was drawn for CNP forms and ALP at intervals of 2 wk.

Effects of glucocorticoids. Acute effects of dexamethasone in sheep. The short-term effects of dexamethasone on CNP forms and ALP were compared in lambs and adult sheep. Eight Coopworth ewe lambs (aged 15 wk) and eight adult Coopworth ewes (aged >4 y) received dexamethasone (125 μg/kg) s.c. twice daily for 4 d. Jugular-vein blood samples were collected at 0900 immediately before injections as well as during the 2-d run-in and run-out periods of study.

Effects of dexamethasone on growth velocity in sheep. For studying changes in both CNP forms and growth, 16 ewe lambs (aged 4 wk) were randomly allocated to receive dexamethasone (125 μg/kg s.c. twice daily; n = 8) or control (0.9% saline solution s.c. twice daily; n = 8) for a period of 15 d. Commencing 6 d before and at intervals of 1–3 d during and after treatment, jugular-vein blood was drawn at 0900 (just before any injections) for analysis of CNP forms and ALP. All animals were weighed and the left metacarpal length was measured (vernier calliper) at intervals of 3–6 d throughout the study. All animal studies were approved by the Lincoln University Animal Ethics Committee.

Plasma assays. Blood samples were collected into chilled standard blood collection tubes that contained EDTA (7.5 mg/mL; Vacutainer, Becton Dickinson, Plymouth, UK) and centrifuged at 4°C, and plasma was stored at −20°C before analysis for CNP, NT-proCNP, and ALP (Aeroset analyser; Abbott Laboratories, Abbot Park, IL). Type 1 collagen C-telopeptide (β-CrossLaps) was measured using an Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). All plasma samples from individual sheep were measured in the one assay.

RIA for amino-terminal proCNP. NT-proCNP was assayed as previously described (13) except that a more sensitive primary rabbit antiserum (J39) raised against NT-proCNP (1–15) was used. Peptide standards were made from synthetic human proCNP (1–19), taking into account the purity data supplied (Chiron Technologies Pty Ltd, Melbourne, Australia). This assay has a detection limit of 1 pmol/L (2 SD from zero) and an EC50 of 90 pmol/L. Within- and between-assay coefficients of variations (CVs) were 5.0 and 7.9%, respectively, at 19 pmol/L.

RIA for CNP22. CNP22 was assayed as previously described (14), except that the assay was preincubated with antisera for 22 h before the addition of ^{125}I-labeled CNP (5000 cpm) and incubated for an additional 24 h at 4°C.

Extraction of peptides from lamb growth plate cartilage. Immediately after the lambs were killed (captive bolt), lamb growth plate cartilage was dissected from proximal or distal ends of limb bones (tibia, metatarsal, or metacarpal), quickly frozen on dry ice, and stored at −80°C before extraction. Frozen cartilaginous tissue (0.5–1 g) was diced, boiled, acidified with acetic acid, and homogenized before extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, MA) as previously described for ovine pituitary tissue (14). Extracts were resuspended in either assay buffer for RIA or 20% CH$_3$CN in 0.1% TFA before size exclusion (Toyo Soda G3000) HPLC. Fetal lamb tissue was obtained after a pregnant ewe was killed using sodium pentobarbital.

Statistical methods. Data are presented as mean ± SEM where appropriate. CVs are presented as percentages and represent the root mean square of CVs from human and lamb serial measurements. *t* test was used to analyze differences in hormone levels between adults and children. ANOVA with repeated measures was used to assess changes in biochemical and physical measurements in lambs using time, sex, and dexamethasone treatment as the independent variables. When significant changes were observed with ANOVA, Bonferroni *post hoc* analysis was used to detect differences from baseline values (day −2) and control time-matched data as appropriate. General linear models were used to test the associations (correlations) between variables using data obtained during weeks 1–19. These were used to test the pooled associations between variables within each lamb. Statistical significance was assumed at $p < 0.05$.

RESULTS

Studies both in adult humans and in lambs showed that plasma levels of NT-proCNP varied little from day to day (CV on repeated sampling from eight lambs over 9 d was 9.3%; range 4–14%). In healthy adult humans (n = 6), NT-proCNP levels drawn at 0900, 1200, 1300, and 1500 showed no significant difference and were not affected by a standard meal ingested at noon (CV = 6.9%; range 3–10%).

Effects of age on plasma CNP forms in humans. Plasma concentrations of NT-proCNP and CNP were measured in humans who ranged in age from 0 to 80 y. As shown in Fig. 1A, plasma NT-proCNP levels in children (5–18 y) were >2-fold those of adults >20 y (mean 46.6 ± 1.7 *versus* 19.2 ± 0.4 pmol/L; $p < 0.001$). Plasma CNP levels were much lower than NT-proCNP levels and showed a small but significant difference between the two groups (mean 1.03 ± 0.05 *versus* 0.93 ± 0.03 pmol/L in children and adults, respectively; $p = 0.045$). Two girls who had recently started on inductive chemotherapy and prednisone (45 and 70 mg/day, respectively) for ALL had extremely low levels of NT-proCNP (9.4 pmol/L at age 10 and 6.7 pmol/L at age 14 y, respectively; Fig. 1A), raising the possibility that high-dose glucocorticoids or chemotherapy suppresses CNP synthesis.

Figure 1. (*A*) Dot plot of NT-proCNP (●) and CNP (○) and mean ± SEM bars in children (aged 5–18 y) compared with levels in healthy adult subjects (aged 20–80 y). Plasma levels of NT-proCNP in two children who received acute chemotherapy and high-dose glucocorticoids for ALL are also shown. (*B*) Plasma NT-proCNP levels (bars ± SEM) stratified by age. Numerals above bars indicate numbers of subjects. Prem, premature (<32 wk) delivery; Term, full-term delivery.

Plasma NT-proCNP levels were elevated at birth and fell progressively with age (Fig. 1*B*). The trend for high levels in cord plasma in premature (mean 193 ± 20 pmol/L; $n = 8$) compared with term deliveries (mean 141 ± 7 pmol/L; $n = 5$) did not attain statistical significance ($p = 0.07$). As shown in Fig. 2*A* and *B*, there was a significant positive association in the 5- to 18-y age group between height velocity and plasma NT-proCNP ($r = 0.57, p = 0.005$) and between ALP (a marker

of bone formation) and plasma NT-proCNP ($r = 0.55, p < 0.001$). The marker of bone resorption type 1 collagen C telopeptide was significantly correlated with NT-proCNP ($r = 0.33, p = 0.013$). Both type 1 collagen C telopeptide ($r = 0.52, p = 0.012$) and ALP ($r = 0.65, p < 0.001$) were significantly correlated with height velocity and each other ($r = 0.67, p < 0.001$).

Changes in plasma CNP forms, ALP, and metacarpal growth in growing lambs. Sequential changes in plasma NT-proCNP, CNP, and ALP in growing lambs from age 1–30 wk are shown in Fig. 3. Levels of all three analytes declined progressively with time ($p < 0.001$ for each) to achieve more stable levels at 15–30 wk. Small increments in NT-proCNP, CNP, and ALP (as well as metacarpal growth velocity; data not shown) occurred in both sexes at 20–25 wk (Fig. 3). A statistically significant effect of sex on levels of NT-proCNP (but not CNP or ALP) was identified with higher mean NT-proCNP concentrations in ram lambs ($F = 8.2, p = 0.015$). Molar concentrations of NT-proCNP were 10- to 15-fold those of CNP throughout the study period.

As shown in Fig. 2*C* and *D*, there was a highly significant positive association of NT-proCNP with ALP ($r = 0.94, p < 0.001$; Fig. 2*C*) and with metacarpal growth velocity ($r = 0.55, p < 0.001$; Fig. 2*D*). Similarly, there was a positive association of metacarpal growth velocity with CNP ($r = 0.48, p < 0.001$) and with ALP ($r = 0.57, p < 0.001$).

Effects of glucocorticoids. The finding of strikingly low levels of NT-proCNP in two children who received inductive chemotherapy and high-dose glucocorticoids prompted study of the effects of dexamethasone on CNP forms and growth velocity in sheep. As shown in Fig. 4, dexamethasone 0.25 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} for 4 d markedly reduced plasma NT-proCNP and ALP in lambs but less so in adult sheep. This differential response to dexamethasone was highly significant for both NT-proCNP ($F = 5.4, p < 0.001$) and ALP ($F = 4.1, p = 0.002$). After 48 h of dexamethasone treatment, plasma NT-proCNP in lambs fell 30.8 ± 3.5% from basal compared with 17.7 ± 2.7% in adult sheep. Both the onset and the offset of dexamethasone's action on NT-proCNP preceded that of ALP (Fig. 4).

To study a possible linkage between changes in CNP and growth velocity, we undertook a more prolonged study in younger growing lambs. As shown in Fig. 5, dexamethasone 0.25 mg $\cdot$ kg^{-1} $\cdot$ d^{-1} for 15 d in 4-wk-old lambs was associated with a highly significant fall in NT-proCNP within 24 h of starting treatment ($F = 7.5, p < 0.001$) and was sustained
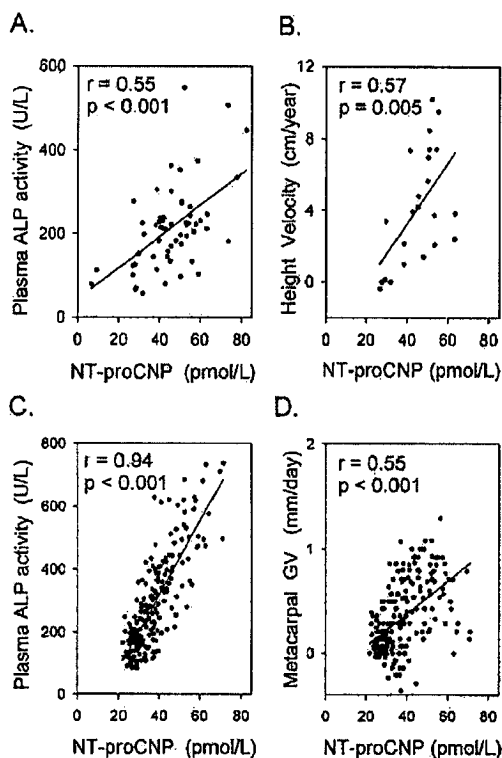

Figure 2. (*A* and *B*) Correlations between plasma NT-proCNP in children (aged 5–18 y) and plasma ALP activity (*A*) and height velocity (*B*). (*C* and *D*) Correlations between plasma NT-proCNP in growing lambs and plasma ALP activity (*C*) and metacarpal growth velocity (GV; *D*).
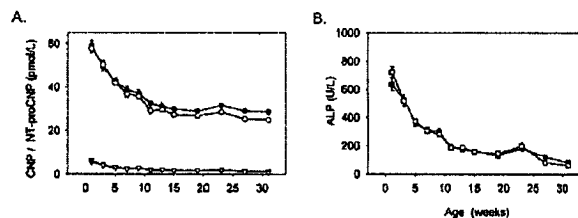

Figure 3. Plasma levels of NT-proCNP (circles) CNP (triangles; *A*) and ALP activity (squares; *B*) in lambs from age 1–30 wk. Rams ($n = 12$, filled symbols); ewes ($n = 12$, open symbols). Values are mean ± SEM.
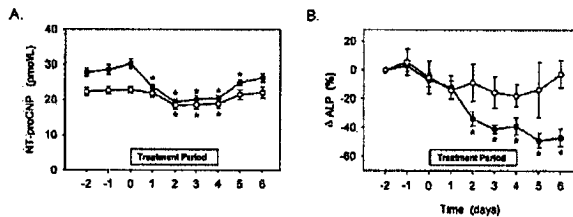
Figure 4. Effect of 4 d of treatment with dexamethasone $(0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})$ in 12-wk-old ewe lambs (●; $n = 8$) and ewe adults (O; $n = 8$) on plasma NT-proCNP (A) and change (Δ; B) in plasma ALP activity. Values are mean ± SEM. Significant differences from baseline values (day −2) are indicated by asterisks (*$p < 0.05$).
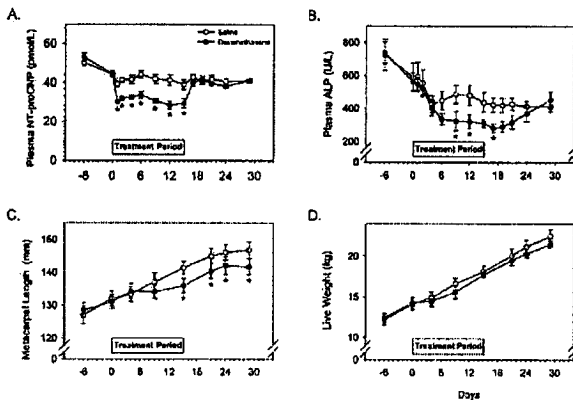

Figure 5. Effect of dexamethasone (●; $n = 8$) or saline control (O; $n = 8$), administered to 4-wk-old ewe lambs for 15 d, on plasma NT-proCNP (A), plasma ALP activity (B), metacarpal length (C), and body weight (D). Values are mean ± SEM. Significant differences between dexamethasone and saline control time-matched data are indicated by asterisks (*$p < 0.05$).

throughout the period of dexamethasone treatment. Similar changes occurred in plasma CNP ($F = 9.2, p < 0.001$; data not shown). Both analytes returned to control levels within 24 h of cessation of treatment. Whereas plasma ALP activity also decreased during the treatment period ($F = 1.9, p = 0.029$; Fig. 5B), the onset and the offset of response in ALP to dexamethasone was delayed when compared with that of NT-proCNP. Dexamethasone treatment was associated with a pronounced decrease in metacarpal elongation ($F = 7.0, p < 0.001$, Fig. 5C), which abated after restoration of NT-proCNP levels.

Compared with saline-treated lambs, a small but significant effect of dexamethasone on body weight ($F = 4.4, p < 0.001$; Fig. 5D) was observed. However, in contrast to metacarpal elongation, differences between treatment groups at cessation of dexamethasone treatment (day 15) were not significantly different.

NT-proCNP in fetal and lamb growth plates. As shown in Fig. 6, NT-proCNP was identified in extracts of tibial growth plate tissue excised from the fetal lamb (18 wk gestation). When subjected to SE-HPLC/RIA, a major peak of immunoreactivity eluted across fractions 29 and 30 (molecular mass ~5 kD). There was no evidence of the prohormone (proCNP $M_r = 10.9$ kD).
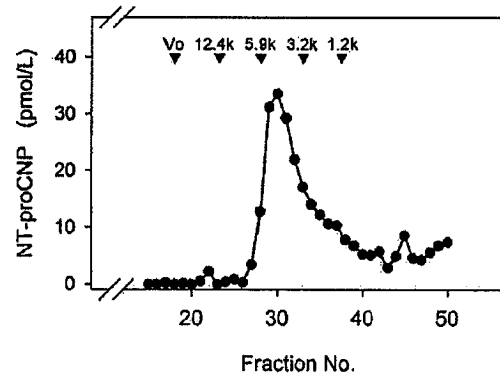

Figure 6. NT-proCNP SE-HPLC immunoreactive profile of growth plate cartilage extract obtained from a fetal lamb tibia (gestation ~18 wk). Column void volume (V_o) and elution positions of molecular markers are shown by arrows.

The concentrations of CNP and NT-proCNP in tissue extracts of limb bone cartilaginous growth plates that were removed from 12-wk-old lambs ($n = 4$) are shown in Table 1 together with blood plasma concentration of these peptides at the time of growth plate collection. Excepting marrow tissue, the concentration (fmol/g) of growth plate extracts exceeded the corresponding level in plasma. Ratios of NT-proCNP to CNP (mean 4; range 1–10) in growth plate tissues were lower than the ratio found in plasma (mean 23; range 21–25).

DISCUSSION

Our results, in showing a strong association of plasma NT-proCNP with growth velocity and markers of bone formation in growing lambs and children, provide support for the hypothesis that plasma NT-proCNP may serve as a marker of growth plate cartilage activity. In keeping with increasing evidence of CNP's role in lengthening the appendicular and axial skeleton (15), the findings now call for prospective studies on the use of plasma NT-proCNP measurements in predicting growth and skeletal development in humans.

As reported previously, plasma concentrations of the biologically active (carboxyterminal) forms of proCNP (CNP 53 and CNP 22) are low in both humans and sheep, close to the

Table 1. *Growth plate tissue, marrow and plasma levels of NT-proCNP, CNP and ratios in 12 week-old ewe lambs (mean + SEM, n = 4)*

Tissue	NT-proCNP (fmol/g)	CNP (fmol/g)	Ratio NT-proCNP/CNP
Tibia			
Proximal	72 ± 13	11 ± 3	6.9 ± 1.2
Distal	48 ± 23	14 ± 7	3.4 ± 0.7
Metatarsal			
Proximal	114 ± 28	57 ± 29	3.2 ± 1.0
Distal	73 ± 31	30 ± 17	2.3 ± 0.6
Metacarpal			
Proximal	89 ± 10	33 ± 3	2.7 ± 0.1
Distal	84 ± 27	29 ± 10	2.7 ± 0.5
Marrow (metatarsal)	23 ± 2	6.6 ± 0.2	3.4 ± 0.2
Plasma (pmol/L)	32 ± 1	1.8 ± 0.1	22.8 ± 0.8

level of the assay detection, and show relatively small changes in pathologic states (2,16). By contrast, NT-proCNP is readily detected and circulates at levels 10- to 50-fold those of CNP. We show here that both NT-proCNP and CNP (despite the low levels) vary inversely with age during the growing period. Using a human chondrosarcoma line, it was shown recently that processing of proCNP (yielding amino-terminal proCNP 1–50 and CNP 53) occurs intracellularly (17). Assuming that equimolar concentrations of amino- and carboxyterminal forms are secreted from cells, rapid uptake and degradation of CNP 53 (or CNP 22) by the natriuretic peptide clearance receptor (NPR-C) and/or hydrolysis by tissue neutral endopeptidase (NEP) (18) will limit access of CNP (but presumably not NT-proCNP) to the circulation. In addition, both degradation pathways will shorten the half-life of CNP in plasma (3). Consistent with this view is the higher ratio of NT-proCNP/CNP in plasma compared with those that we found in growth plate cartilage (Table 1). This differential vulnerability of CNP forms to degradation by NPR-C and NEP, both of which are expressed in cartilage and skeletal tissue (19,20), increasing the likelihood that plasma NT-proCNP will better reflect CNP synthesis within tissues provided that renal function is normal (16). To obtain more direct evidence that growth plate cartilage levels were enriched (compared with jugular venous plasma), we sampled a variety of growth plate cartilage tissue extracts in 12-wk-old lambs. Although tissue levels were higher than found in plasma, it should be noted that this study was undertaken at a time when plasma levels were close to mature (adult) levels and when linear growth was almost complete. Further studies are planned in younger, rapidly growing lambs to define the linkage, if any, among levels of NT-proCNP in growth plates, chondrocyte immunohistochemistry, and growth velocity. Presumably a number of tissues contribute to circulating levels of NT-proCNP. CNP gene expression in rodents is reported in a variety of extraskeletal tissues, including brain, reproductive tissues, lung, and vascular endothelium (21–24). All of these tissues are potential sources of circulating NT-proCNP. However, our finding of strong associations of blood levels with growth velocity and markers of bone growth, together with the close temporal relationship of NT-proCNP with changes in metacarpal growth and ALP during glucocorticoid treatment, point to an important contribution from growing cartilage. Although we cannot exclude a placental contribution to the very high levels of NT-proCNP found in cord plasma, previous work (25) showing lower molar concentrations of NT-proCNP in placental tissues than in cord plasma and evidence that natriuretic peptides (ANP) do not cross the placenta (26) make this unlikely. It remains to be seen whether cartilage, bone, or other (possibly vascular) tissues contribute to the relatively stable levels of NT-proCNP that we observe in mature normal adults (humans) and sheep. However, the small fall in levels (~17%) in adult sheep during short-term dexamethasone treatment (0.25 mg · kg^{-1} · d^{-1}) is consistent with some sustained contribution from the skeleton even in the fully mature state.

Although the duration of linear growth is different in the two species (~1.5 y in sheep, up to 18 y in humans), the pattern of decrease in plasma NT-proCNP (from birth to maturity) seems to be similar and is consistent with the progressive decrease in hypertrophic and proliferative chondrocytes found in human

studies (27). It is interesting that higher levels were noted in males [rams in current study and in humans (16)], in contrast to the consistently higher levels of ANP/BNP and related forms in females (28). Similar gender effects are reported for markers of collagen 1 (29) and collagen 3 (30) synthesis, which have been attributed to greater bone volumes in males. Our more recent analysis of cord plasma NT-proCNP in preterm and term infants ($n = 23$) shows a strong negative relationship of level with gestational age ($r = -0.49, p = 0.015$). Similar negative associations have been found for markers of collagen 1 and collagen 3 turnover with gestational age (30). These relationships are consistent with linkage of both cartilage activity and bone turnover with growth velocity, which is maximum in the human fetus during the third trimester and in the first year of postnatal life. A much more defined database is now required, relating NT-proCNP level to age and concurrent growth velocity throughout the growing period in normal children and in adolescents. Our study was not designed to examine changes in the peripubertal growth phase, which would require more frequent and focused sampling commencing at the onset of the pubertal growth surge. Although nothing is known of changes in CNP gene expression during this phase of growth, it is to be noted that small increments in NT-proCNP and ALP were found in both sexes at 20–25 wk in lambs. Conceivably, a short period of increased CNP synthesis within key growth plates underlies these changes. It is interesting that in rodent models of impaired CNP gene expression (9), as well as in loss of function mutations of the human NPR-B (10), linear growth impairment is not fully expressed until after birth. It remains to be seen whether plasma NT-proCNP levels during the first year of life in humans predict final adult height and whether cord plasma levels have an impact on growth rate or skeletal development as previously reported for IGF-1 (31) or calcium concentration (32).

The finding of very low levels of NT-proCNP (the lowest that we have observed in our laboratory) in two children who recently started on chemotherapy and high doses of glucocorticoids prompted study of dexamethasone's action in lambs and adult sheep. As expected from previous work showing glucocorticoid-induced inhibition of chondrocyte proliferation and differentiation (33), we noted prompt and significant decreases in both NT-proCNP and ALP with dexamethasone administration, effects that were greater in lambs than in mature adult sheep in keeping with the larger content of growth plate cartilage in lambs. That these changes are associated with linear growth inhibition was shown by a longer period of dexamethasone treatment in younger lambs. In both short- and long-term studies, the onset and the offset of dexamethasone's effect on NT-proCNP preceded that observed in ALP. Although plasma ALP in lambs is presumably sourced from osteoblasts and other tissues, as well as cartilage, this differential effect is consistent with actions of dexamethasone early in the cell cycle (*e.g.* on proliferating chondrocytes) with consequential (delayed) effects on the later maturing hypertrophic zone from which ALP (in cartilage) is largely sourced (34). It is to be noted that normal levels of plasma NT-proCNP were restored within 24 h after cessation of dexamethasone, whereas ALP did not attain "control" levels for an additional

9 d, during which time the inhibitory effect on metacarpal lengthening had abated. The precise mechanism whereby glucocorticoids inhibit NT-proCNP was not addressed in our study. Possibly, levels fall as a consequence of depletion of proliferative chondrocytes (35) from which CNP is largely sourced (9). Although a classic glucocorticoid response sequence has not been identified in the CNP gene (36), inhibitory effects of glucocorticoids may be mediated by changes in transcriptional factors acting at the level of the CNP promoter. In this context, it is of note that in rat articular chondrocytes (37), the signalling pathway of TGF-β, a potent stimulus to CNP mRNA expression (22,38), is blocked by dexamethasone. TGF-β is known to up-regulate CNP expression through the transcriptional modulator TSC-22, which binds to GC-rich cis elements within the CNP promoter (38). Similar inhibitory effects of dexamethasone on TGF-β synthesis (39) or signalling (40) have been described in other cell systems, raising the possibility that the growth arrest results in part from loss of CNP and its trophic action on chondrocytes (41) and on matrix synthesis (42). If proved, then such a mechanism of action could have important therapeutic implications, including the prospect of interventions that restore (42) or maintain (43) local levels of CNP within the growth plate and reduce the deleterious effects of glucocorticoids on the growing skeleton. The daily doses of glucocorticoid used here (approximately equivalent to 35–40 mg/d prednisone in a 30-kg child) are less than the doses received by children with ALL (45–75 mg/d prednisone). Chemotherapy also has the potential to inhibit chondrocyte proliferation and linear height (44) and also may have contributed to the profoundly low values observed in the two children. Clearly, the separate effects of glucocorticoid and chemotherapies at a range of doses are required to clarify the actions of these drugs.

Conclusions drawn from our findings need to be tempered in light of several limitations. Although the associations of plasma NT-proCNP with growth velocity and bone markers are strong, proof that levels truly reflect changes in growth plate activity requires further study, possibly using genetic models in which the expression of CNP within cartilage is rendered dysfunctional. Furthermore, it is well recognized that glucocorticoids have profound inhibitory effects on protein synthesis in many tissues, not just growth plate chondrocytes. Thus, it is possible that the changes that we observed reflect inhibition of CNP gene expression in several different tissues. Finally, our studies in growing children were necessarily limited in several respects: only a single value (cross-sectional study) was obtained in any one child; a range of different morbid states and treatments were represented in this study group (which did not include children in the postnatal to 5 y age group), and the numbers of children in whom it was possible to calculate growth velocity was less than half the original study population. Despite these limitations, the findings drawn from cord blood samples and children in the 5- to 18-y group are consistent with those observed in healthy growing lambs.

Should future studies confirm that plasma NT-proCNP is largely sourced from cartilage in the developing skeleton, monitoring the level could be a valuable aid to predicting future growth potential as well as helpful in providing an early warning of impaired growth plate activity in patients who receive glucocorticoids or other agents that are known to affect growth adversely. Conceivably, genetic disorders of CNP synthesis and/or action may be identified by abnormality of plasma NT-proCNP once an age-related reference range is established and the contribution of other organ dysfunctions (e.g. renal impairment) is more fully defined. In conclusion, NT-proCNP levels in blood show a strong association with growth velocity and markers of bone formation and may well serve as a useful marker of cartilage growth plate activity and health.

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